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Electromagnetic Biology and Medicine

ISSN: 1536-8378 (Print) 1536-8386 (Online) Journal homepage: http://www.tandfonline.com/loi/iebm20

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To cite this article: Timur Saliev, Dinara M. Baiskhanova, Alma Akhmetova, Dinara A. Begimbetova, Mars Akishev, Gulsim Kulsharova, Askhat Molkenov, Talgat Nurgozhin, Tatiana Alekseyeva & Sergey Mikhalovsky (2018): Impact of electromagnetic fields on *in vitro* toxicity of silver and graphene nanoparticles, Electromagnetic Biology and Medicine, DOI: 10.1080/15368378.2018.1534740

To link to this article: https://doi.org/10.1080/15368378.2018.1534740

Published online: 08 Nov 2018.

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ELECTROMAGNETIC BIOLOGY AND MEDICINE
https://doi.org/10.1080/15368378.2018.1534740

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Impact of electromagnetic fields on *in vitro* toxicity of silver and graphene nanoparticles

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ABSTRACT

The correlation between shape and concentration of silver nanoparticles (AgNPs), their cytotoxicity and formation of reactive oxygen species (ROS) in the presence of electromagnetic fields (EMFs) has been investigated. In addition, the bio-effects caused by the combination of EMFs and graphene nanoparticles (GrNPs) have been also assessed. The AgNPs of three shapes (triangular, spherical and colloidal) and GrNPs were added in high concentrations to the culture of human fibroblasts and exposed to EMF of three different frequencies: 900, 2400 and 7500 MHz. The results demonstrated the dependence of the EMF-induced cytotoxicity on the shape and concentration of AgNPs. The maximal cell killing effect was observed at 900 MHz frequency for NPs of all shapes and concentrations. The highest temperature elevation was observed for GrNPs solution irradiated by EMF of 900 MHz frequency. The exposure to EMF led to significant increase of ROS formation in triangular and colloidal AgNPs solutions. However, no impact of EMF on ROS production was detected for spherical AgNPs. GrNPs demonstrated ROS-protective activity that was dependent on their concentration. Our findings indicate the feasibility to control cytotoxicity of AgNPs by means of EMFs. The effect EMF on the biological activity of AgNPs and GrNPs is reported here for the first time.

ARTICLE HISTORY
Received 18 May 2018
Accepted 7 October 2018

KEYWORDS
Electromagnetic field; silver nanoparticles; graphene; reactive oxygen species; toxicity; fibroblasts

Introduction

Silver-based materials have been utilized extensively in biomedicine and industry due to their unique chemical and physical properties. First of all, there is the proven antimicrobial action of silver ions against a range of various pathogens. Silver-containing materials have been actively utilized for personal care and household products, food protection, textile and construction materials (Rajeshkumar and Malarkodi, 2014; Srisod et al., 2018). The medical applications of silver have been expanded, but not limited, to the following areas: incorporation of silver species in antibacterial creams, eye drops, wound dressings and coating of medical/surgical devices. The recent progress in nanotechnology opens the perspectives for manufacturing of silver nanoparticles (AgNPs) of different shapes and sizes. Such a technology provides an opportunity to enlarge the surface area of silver without increasing its weight leading to an amplification of antimicrobial action. In addition, the use of nanosized particles results in deeper penetration, thus reaching the remote sites of the organism and overcoming the natural barriers between tissues and cells.

The application of AgNPs has been quickly adopted for molecular medicine, diagnostic and therapeutic applications (Pantic, 2014; Wong and Liu, 2010). AgNPs have been employed for metal-enhanced fluorescence for RNA/DNA hybridization (Sabanayagam and Lakowicz, 2007), microwave-accelerated metal-enhanced fluorescence (Aslan et al., 2006), chemiluminescence assays (C. H. Liu et al., 2006), AgNP-based surface-enhanced Raman spectroscopy (Zhang et al., 2011), and electrochemical detection (Niu et al., 2009).

Apart from a huge potential for bio-sensing and imaging, the mainstream use of AgNPs remains in antibacterial applications. Many reports have demonstrated antimicrobial and anti-viral action of AgNPs against a range of gram-positive and gram-negative bacteria, including *Staphylococcus aureus* (Dehkordi et al., 2011; Mirzajani et al., 2011), *Streptococcus* (Espinosa-Cristobal et al., 2009), *Escherichia coli* (Sondi and Salopek-Sondi,

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2004), *Pseudomonas aeruginosa* (Guzman et al., 2012), *Klebsiella pneumoniae* (Rajeshkumar and Malarkodi, 2014) and viruses (Lu et al., 2008). It follows logically that AgNPs have been recruited to fight wound infections, including antibiotic-resistant strains (Kasithevar et al., 2017; Williams et al., 2017). Antibacterial activity of AgNPs has been also intensively exploited in dentistry for implant coatings, suppression of fungal pathogens and endodontic bacteria, local anesthesia and treatment of oral cancer (Bapat et al., 2018; Noronha et al., 2017). Aside from oral cancer, there is also a range of studies investigating a possible application of AgNPs for treatment of various forms of tumors (Choi et al., 2018; Jeyaraj et al., 2013; Nakkala et al., 2018; Yuan et al., 2017).

However, the mechanism of antimicrobial action of AgNPs is still a topic of discussion. There are three main theories explaining their bactericidal activity: direct damage of bacterial membrane (Sondi and Salopek-Sondi, 2004), formation of reactive oxygen species (ROS) (Xu et al., 2012) and release of silver ions (Ninganagouda et al., 2014; Onodera et al., 2015). It has been demonstrated that the silver ions directly affect the DNA replication processes, and they are capable of inactivating some vital bacterial enzymes (Arakawa et al., 2001; Matsumura et al., 2003). Other reports suggest that the formation of ROS is the main contributing factor to the antibacterial action of silver ions and AgNPs (Holt and Bard, 2005; H. J. Park et al., 2009). Mukherjee et al. (2014) hypothesized that silver ions released from AgNPs interact with cellular glutathione followed by its oxidation. It results in excessive production of ROS and lipid peroxidation, which, in turn, leads to bacterial inhibition.

It has been also shown that the cytotoxicity and production of ROS depend on the size of AgNPs (Onodera et al., 2015; Soares et al., 2015) and their concentration (Sengstock et al., 2014). In fact, the influence of AgNPs size on their cytotoxicity was demonstrated in the range of studies (Alqahtani et al., 2016; Bouallegui et al., 2018; Cho et al., 2018; Makama et al., 2018).

W. Liu et al. (2010) demonstrated that the cytotoxic effect and ROS formation induced by AgNPs was size-dependent. Albers et al. (2013) also showed the dependence of cytotoxicity of AgNPs on their size and concentrations. The strongest cytotoxic effect was found for AgNPs with the size of 50 nm, and the weakest effect for 3 μm particles. As anticipated, the correlation between the AgNP's concentration, decrease of cell viability and increase of antibacterial activity was demonstrated. In another study, Habas et al. (2018) scrutinized the mechanism of cytotoxic and genotoxic effects induced by AgNPs at different concentrations on a primary culture of mouse Sertoli cells (*in vitro*). Their results showed that AgNPs were able of increasing oxidative stress levels along with the activation of p53, repression of bcl-2 protein and reduction of endogenous antioxidant enzymes. Shukla et al. (2016) reported on a size-controlled green synthesis of resveratrol-stabilized AgNPs. The fabricated resveratrol-capped AgNPs were exceptionally stable for months and exhibited a high antibacterial activity against gram-negative wild-type *E. coli* (type BW 25113).

Apart from the size, it has been proposed that the NPs' shape may also affect their chemical and biological properties. Pal et al. (2007) investigated the antibacterial activity of AgNPs of different shapes against bacteria *E. coli*. The strongest biocidal action was observed for AgNPs of triangular shape comparing to spherical and rod-shaped NPs.

Hao and Schatz (2004) reported on the impact of AgNPs shape on induction of electromagnetic fields (EMFs) around AgNPs exposed to optical excitation of localized surface plasmon resonances. It has been suggested that at the plasmon resonant wavelength, AgNPs could exhibit properties of nano-antennae thus elevating the intensity of the ambient EMF. The plasmonic behavior of size-selected supported silver clusters was studied by Lunskens with coauthors who used surface second harmonic generation spectroscopy (Lunskens et al., 2015, 2017). F. Liu and Nunzi (2012) reported on surface plasmon effects in different organic optoelectronic devices. Jakob et al. (2018) synthesized water-soluble ligand-protected optically active silver nanostructures. The synthesis was performed successfully with D- and L-cysteine and L-glutathione.

Despite abovementioned reports, there is lack of information on how the shape of AgNPs affects formation of ROS. Moreover, little information is known about an impact of EMF on ROS production triggered by AgNPs in living systems. To address aforementioned issues, we studied the bio-effects induced by the combination of AgNPs and EMF. The studies encompassed the determination of cytotoxicity, production of ROS and thermal activity in relation to the shape and concentration of NPs. This information is a prerequisite for developing a novel method of wound treatment, where AgNPs will be incorporated into wound dressing followed by their activation by external application of EMF.

Another promising bio-nanomaterial with exceptional optical, electrical and chemical properties is graphene. In parallel to intensive research and attempts to harness unique features of graphene for industrial applications, some groups started exploring the potential of utilizing graphene for biomedical purposes (Z. Liu et al., 2008; Sun et al., 2008). The earliest studies have been predominantly focused on anticancer therapy and drug delivery (Jokar et al., 2014; X. Y. Yang

et al., 2011; L. M. Zhang et al., 2010a; Zuchowska et al., 2017). Later on, the interest in biomedical application of graphene has been expanded to imaging (Chowdhury et al., 2013; Lalwani et al., 2013; H. Zhao et al., 2017), bio-sensing (Hernandez-Sanchez et al., 2018; D. W. Park et al., 2014a; Tong et al., 2018) and tissue engineering (Bai et al., 2018; Goenka et al., 2014). Despite a great biomedical potential, high biocompatibility, low cytotoxicity and genotoxicity, performance of graphene and its derivatives in biomedical applications are poorly understood and seldom described in the current literature (Mahanta and Paul, 2015; Mullick Chowdhury et al., 2013; Y. Zhang et al., 2010b). In particular, there is lack of information on the correlation between graphene concentration, its cytotoxicity and EMFs. To address this issue, we investigated the interaction between EMFs, graphene nanoparticles (GrNPs) and human cells.

Materials and methods

Cell culture and determination of cell viability and ROS

Human fibroblasts have been used in the experiments. The fibroblasts were isolated from a child's foreskin after circumcision (donated by child's parents). The isolation and culturing human fibroblasts have been done according to the method described previously (Rittie and Fisher, 2005). The fibroblasts were seeded onto the surface of a 96-well culture plate (Corning 96-Well Multiwell Plate, Sigma-Aldrich/Merck, USA) at the density of 10×10^3 cells/well prior to the exposure by EMF and NPs. A combination of two fluorescent dyes has been employed for cytotoxicity tests: propidium iodide (PI) and DAPI (4′,6-diamidino-2-phenylindole). PI cell staining indicates necrotic cell death, whilst DAPI stains both live and dead cells. The samples were analyzed using a highly sensitive multi-hybrid reader Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, USA). The number of viable cells was calculated by subtracting the amount of necrotic/dead cells from the total cell population.

For determination of ROS level in cell culture treated with EMF, we employed CM-H_2DCFDA dye (Life Technologies, USA). This fluorescent dye is the chloromethyl derivative of H_2DCFDA, and it has been routinely used for detection of ROS, including hydrogen peroxide, hydroxyl radicals and peroxynitrite (Chernyak et al., 2006; Meissner et al., 2008; Samudio et al., 2005). A hydrogen peroxide (H_2O_2) was utilized as a classic apoptosis mediator and an inducer of ROS formation (positive control) (Kajiwara et al., 2001; Kim et al., 2006).

Ethics

All procedures related to isolation of human fibroblasts were performed under the protocols approved by the Ethics Committee of the Centre for Life Sciences of Nazarbayev University (Registration number IORG 0006963). The fibroblasts were isolated from a child's foreskin after circumcision (donated by child's parents). The samples were obtained from National Scientific Medical Centre (Astana, Kazakhstan). A written informed consent was signed by parents prior to the experiments.

AgNPs

For the synthesis of AgNPs, silver nitrate (>99% $AgNO_3$) and sodium borohydride (99% $NaBH_4$) were purchased from Sigma-Aldrich, USA. Tunable, rainbow colored, colloidal AgNPs were synthesized and characterized using single-nanoparticle plasmonic microscopy and spectroscopy ("rainbow method") (Huang and Xu, 2010). Spherical and triangular NPs were obtained utilizing the "rainbow method", where amount of sodium borohydride was found to alter the shape and size of the NPs. The size of AgNPs and graphene has been determined by using a particle size measuring device, Nanotrac Wave (Microtrac, USA). The size of the particles was as follows: triangular AgNPs 12.8 nm (SD ±3.0 nm), spherical AgNPs 8.2 nm (SD ±3.5 nm), and colloidal (mixed morphology) AgNPs 13.4 nm (SD ±5.5 nm) (Figure 1a–c).

UV–visible spectrophotometry, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were employed for characterization of AgNPs. For UV–visible spectrophotometry, PhotoLab 6600 UV–vis spectrophotometre (WTW, Germany) was utilized. SEM and TEM analyses were carried out using Zeiss SIGMA FEG-STEM system (Zeiss, Germany).

GrNPs

GrNPs were obtained by repeated electrochemical intercalation of graphite foil (TU 26.8-30969031-002-2002) under low concentration of alkaline electrolyte solution (KOH), with electrical current density in the range of 6.0–60.0 mA/cm^2. The graphite foil served as an electrode. The NPs were analyzed by Raman scattering method, laser correlation spectroscopy and scanning electron spectroscopy (SEM, JEOL SL6060LA, Japan)

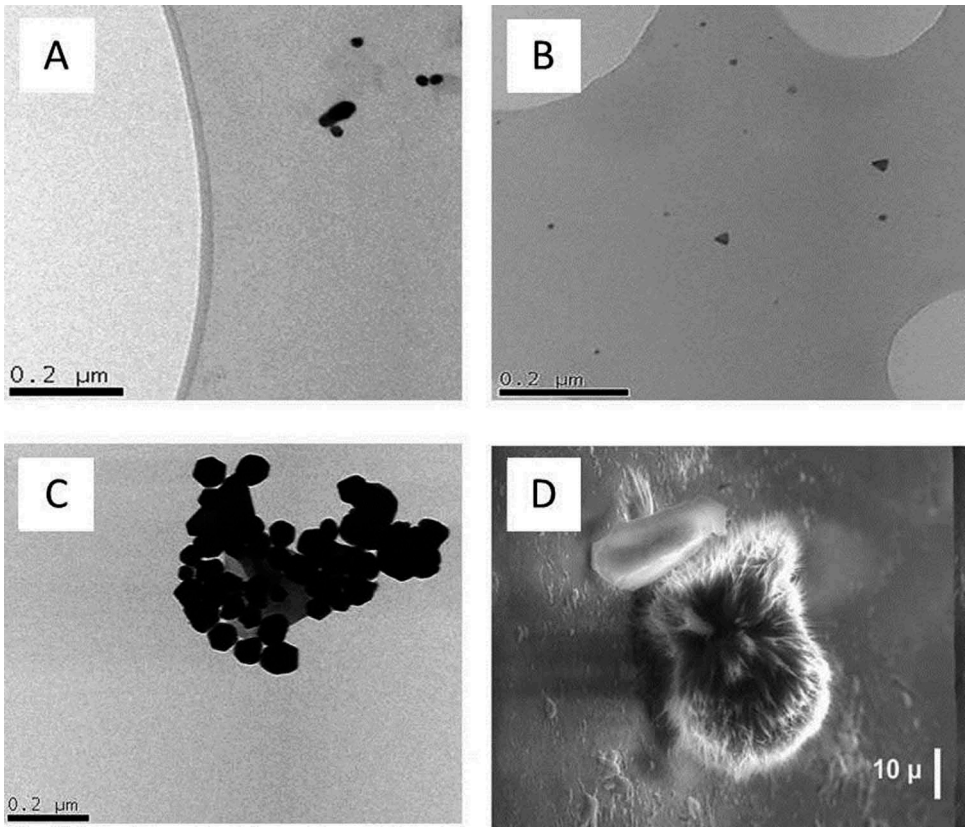

Figure 1. TEM/SEM images of nanoparticles. (A) TEM image of spherical AgNPs, (B) TEM image of triangular AgNPs, (C) TEM image of mixed morphology AgNPs and (D) SEM image of graphene.

(Figure 1d). After electrochemical treatment of the graphite foil and separation from any large particles, the solution was deposited on a gold film substrate, dried and then analyzed by SEM (Figure 1d). The SEM image clearly shows the lamellar structure of the particles.

Setup for EM exposure

The setup for EM exposure included generator of EM signal, inner irradiating antenna, EM spectrum analyzer and a Faraday cage (Rainford PTC JRE 0814 Wireless RF Test Enclosures System; Rainford EMC Systems Ltd, UK). The Faraday cage was used for the protection of cell culture from interrupting ambient EMFs. This system provides complete EM and acoustic isolation. Three frequencies were used in the experiments: 900, 2400 and 7500 MHz. For each frequency, dedicated Lab Brick Signal generators (Vaunix Technology Corporation, USA) were utilized. The exposure was conducted at 10 W peak power pulsed amplitude modulation with a pulse width of 0.6 ms and a repetition rate of 217 Hz. The EM wave-shape was sinusoidal. The EM exposure duration was 10 min.

The reference distances (Fraunhofer distance) from antenna to the sample (96-well culture plate) were calculated through the formula:

$$\text{Far Filed Region} = \frac{2\ (D)^2}{\lambda} \tag{1}$$

where D is the largest dimension of the irradiating antenna and λ is the wavelength of corresponding frequency.

The distance for 900 MHz frequency was 6 cm, for 1800 MHz was 12 cm and for 2400 MHz it was 16 cm. These distances provide a homogenous distribution of EMFs with corresponding frequencies.

The human fibroblasts were seeded onto the surface of a 96-well culture plate (details are provided below) and then exposed to EMF. The irradiation of cell culture was performed inside the Faraday cage (Rainford PTC), which was placed into an incubator with humidified air (5% CO_2). The control group was kept at the same conditions as an experimental one, but without any EM exposure.

The specific absorption rate (SAR) (W/kg) was calculated using the following equation:

$$\text{SAR} = \frac{\sigma|E|^2}{\rho} \tag{2}$$

where E is the peak value of electric field, σ is the conductivity (S/m) and ρ is the mass density of cells (kg/m^3). This method of SAR calculation for cell culture (as a monolayer) was previously described in the literature by McIntosh et al. (2003) and J. X. Zhao (2005). The average SAR on the bottom of the plate was 0.20 W/kg for 900 MHz frequency, 0.40 W/kg for 1800 MHz and 0.53 W/kg for 2400 MHz.

Temperature monitoring

A special calibrated fiber-optic sensor TS3 thermocouple (OPTOCON, Germany) was utilized for temperature monitoring. This type of thermocouple guarantees a stable temperature measurement at EM/Radio frequency environment. "FOTEMP-Assistant" software (OPTOCON, Germany) running on Microsoft Windows operating system was used for temperature monitoring and analysis.

Statistical analysis

The one-factor analysis of variance with repetition (one-way ANOVA; Dunnett's test) was used to analyze the results. Each experiment was conducted at least in triplicate. Statistical analysis was performed using SigmaPlot 11.0 software (Systat Software Inc., California, USA).

Results

Effect of EMFs on cytotoxicity of nanoparticles

The results obtained show that AgNPs of all three shapes and at four different high concentrations (400, 600, 800 and 1000 μg/ml) were not cytotoxic to human fibroblasts (Figure 2a). Nevertheless, the application of EMFs led to the dramatic drop in cell viability. The lowest viability ratio was found out in the samples treated by the combination of colloidal AgNPs and 900 MHz frequency. Observational data from the optical microscopy inspection of treated cells confirm to the results of viability studies. Second by combined cytotoxic effectiveness was the 2400 MHz frequency. A slight rise of viability was detected in the cell population exposed to graphene and EMF of 7500 MHz, indicating a cyto-protective effect of such combination (Figure 3a). The data of observation of treated cells by optical microscopy confirm to the results of viability studies (Figure 4).

Impact of combination of EMF and nanoparticles on formation of ROS

The results demonstrated a correlation between ROS formation and shape of AgNPs (Figure 2b). Moreover, we observed the dependence of bio-effects on AgNPs' concentration and EM frequency. The most effective in terms of generation of ROS were triangular AgNPs followed by colloidal counterparts. Notably, triangular NPs in the highest concentration (1000 μg/ml) were able to trigger ROS formation up to twofold even without EMF exposure (compared to the control). The optimal EM frequency for ROS induction was found to be 7500 MHz. However, no substantial rise of ROS formation in the cell culture treated by combination of spherical AgNPs and EMF was detected.

The obtained data revealed that EM irradiation of the fibroblast culture in the presence of GrNPs suppressed the ROS production at all concentrations and EM frequencies studied (Figure 3b). Moreover, the increase of GrNPs concentration led to a stable decrease of the level of ROS. In terms of suppression of ROS formation, the 7500-MHz frequency was less effective than the other two frequencies (900 and 2400 MHz).

Thermal effects

We did not observe any significant heating effect induced by combination of AgNPs and EMFs (Figure 2c). The maximal heating effect was detected in the samples treated with GrNPs and EMF at 900 MHz frequency (Figure 3c). Interestingly, this effect was consistent for all GrNPs concentrations. These findings might be explained by unique thermal (Pop et al., 2012), electric (Bernad et al., 2010) and magnetic conductivity (C. H. Yang et al., 2010) of graphene.

Discussion

Our findings demonstrated that the AgNPs were not toxic to human fibroblasts at high concentrations and given experimental conditions. These observations are in contradiction to the results of other studies, particular to the work conducted by Gliga et al. (2014). However, it should be pointed out that AgNPs sizes used in the present work (12.8 nm for triangular NPs, 8.2 nm for spherical NPs and 13.4 nm for colloidal NPs) differ from the AgNP sizes utilized by Gliga and coauthors (10, 40 and 75 nm). Moreover, Gliga et al. used polyvinylpyrrolidone coating for NPs sized 10 nm, which may affect chemical stability and biological

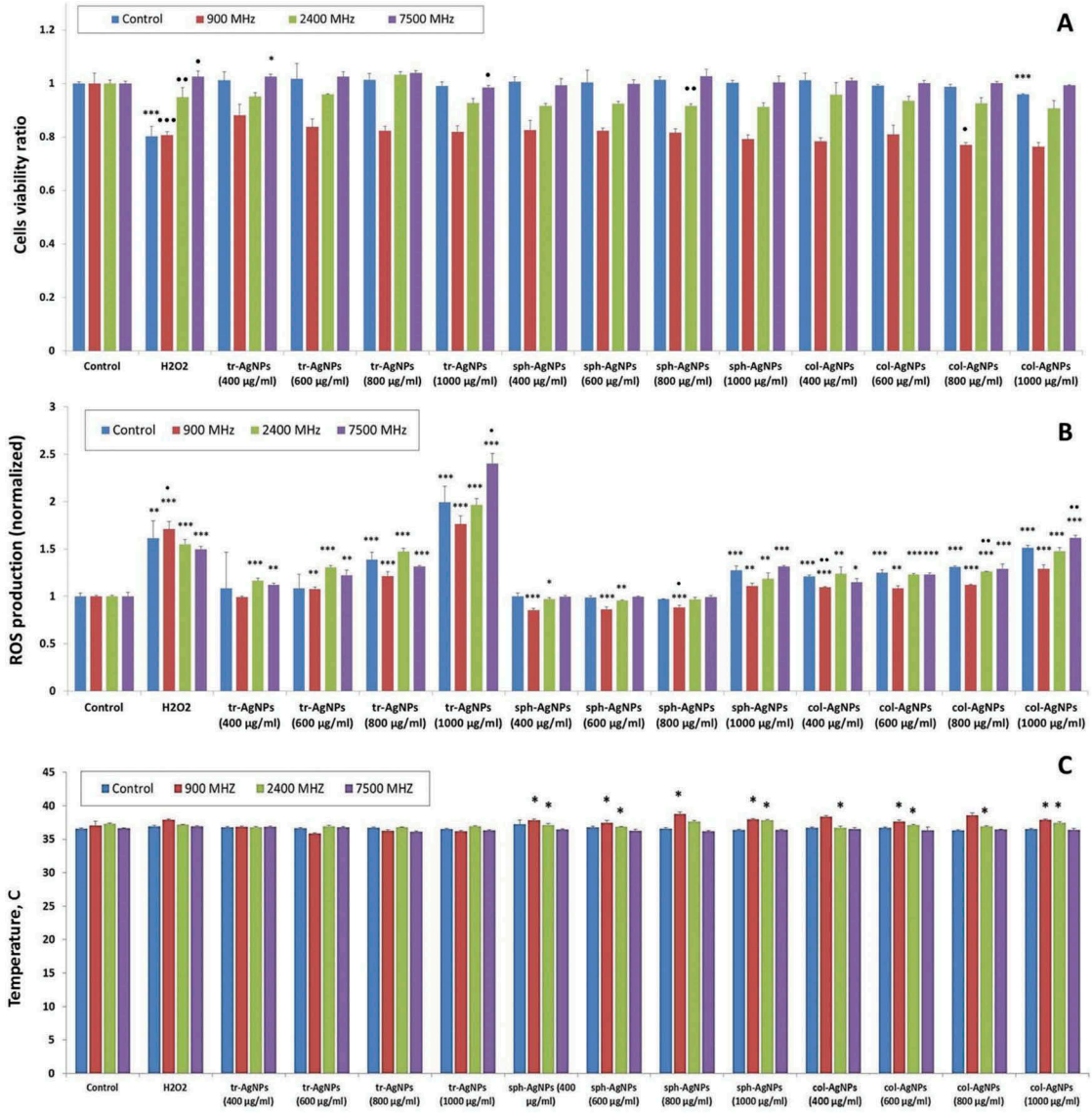

Figure 2. The bio-effects of combination of AgNPs (concentrations 400, 600, 800 and 1000 µg/ml) and EMF (900, 2400 and 7500 MHz). Hydrogen peroxide (H_2O_2) is a positive control. (A) Cell viability normalized ratio, (B) ROS production (normalized ratio to control) and (C) data of temperature monitoring (°C). Bars represent means ($n = 3$). ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$ – compared to main control. ●●●$p \leq 0.001$, ●●$p \leq 0.01$, ●$p \leq 0.05$ – compared to control from the same group (same nanoparticles concentration).

properties of NPs. Therefore, it is highly likely that there is a size and coating-dependent toxicity of AgNPs, based on the size dependence and stability over other biological properties of NPs.

In another study conducted by Park et al. (2010), the toxic effect of AgNPs has been also experimentally shown. However, the duration of treatment in this study was much longer (24, 48, 72 and 96 h) than in our study. Second, the cell model was completely different: Park and coworkers used mouse peritoneal macrophage cell line, while our goal was to test a human cell line. The short time interval in our study was dictated by our intention to develop a system which could be utilized for wound treatment. In this case, the time of EM exposure is highly critical, and we chose to use the minimal period of irradiation to avoid possible negative effects on the organism.

The frequency of 900 MHz was effective in terms of cell killing for all types of particles and concentrations, including GrNPs. In case of graphene, the detected cytotoxicity might be linked to the thermal effect induced by EMF (Figure 3c). We have also observed concentration-dependent cytotoxic effects at the exposure cells to EMF with 900 MHz frequency, when the increase of concentration correlated with cell death, except in the case of added GrNPs. On the other hand, the irradiation of cell culture

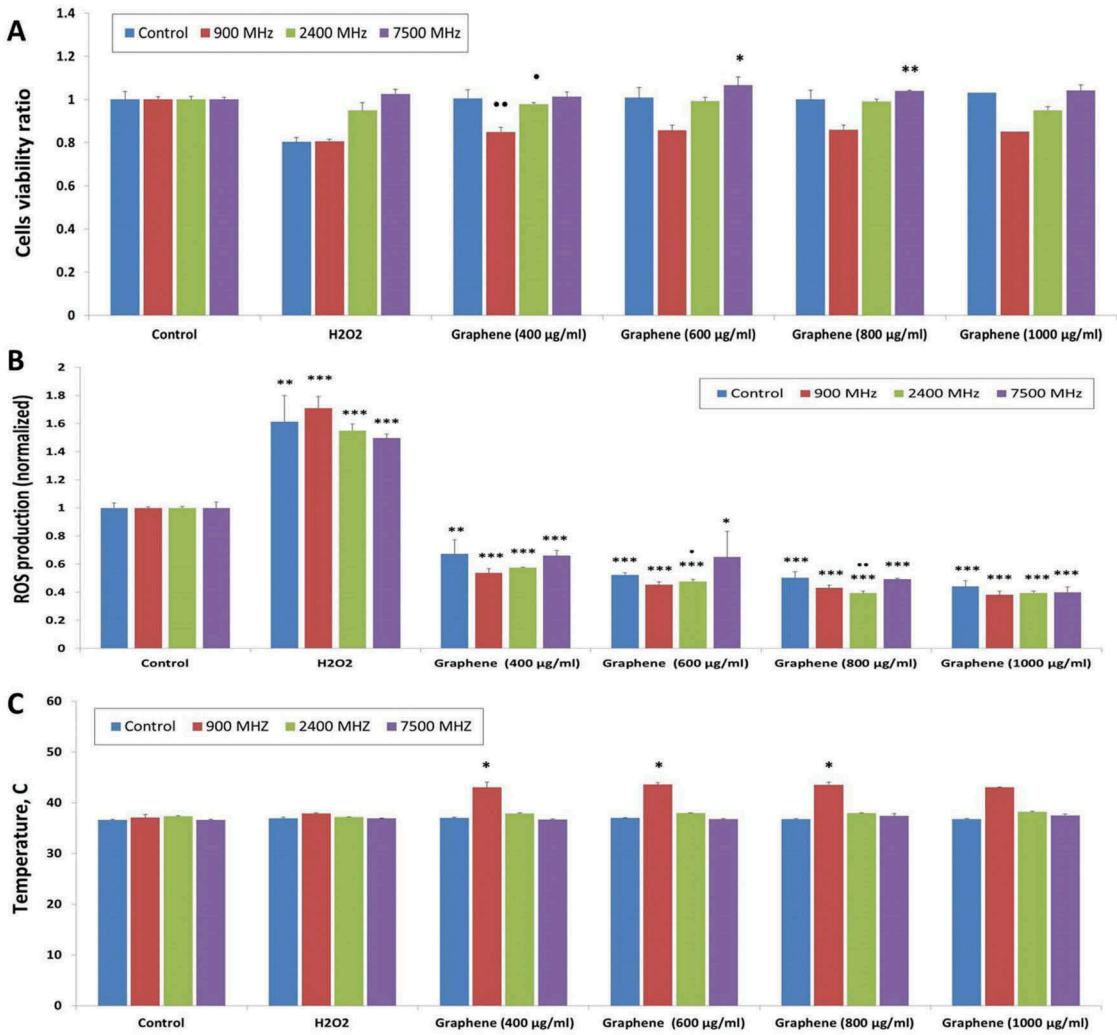

Figure 3. The bio-effects of combination of graphene NPs and EMF. Graphene NPs are presented in four concentrations (400, 600, 800 and 1000 µg/ml). Hydrogen peroxide (H_2O_2) is a positive control. Three EM frequencies were applied: 900, 2400 and 7500 MHz. (A) Cell viability normalized ratio, (B) ROS production (normalized ratio to control) and (C) data of temperature monitoring (°C). Bars represent means ($n = 3$). ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$ – compared to main control. ●●●$p \leq 0.001$, ●●$p \leq 0.01$, ●$p \leq 0.05$ – compared to control from the same group (same graphene concentration).

by the highest frequency (7500 MHz) did not cause any impact on cell viability for all experimental groups. Moreover, a slight increase of cell viability in the culture treated by graphene was detected.

Our findings support the hypothesis that graphene facilitates cell proliferation and confirm to previous reports on the graphene's cell protective effect (Elkhenany et al., 2015; J. Park et al., 2014b). The frequency of 7500 MHz was found to be the most favorable for the cells, when it was employed in the combination with AgNPs and GrNPs. In contrast, 900-MHz frequency was found as the most hostile to the cells in combination with NPs.

We have observed the elevation of ROS level as a result of the combined use of AgNPs and EMF in all experimental groups (except with added GrNPs). The maximum level of ROS was detected in the group treated with the triangular AgNPs. It is well known that there is a huge focusing of the electric field magnitude in the vicinity of a metal spike; so at the triangular apexes, there will be regions of a very high-intensity electric field, which could be very effective at ROS generation. The intensity of electric field in the polar regions of a sphere is much lower, which substantially reduces ROS production.

Such EMF-induced cytotoxicity might have further therapeutic applications, including the suppression of infections caused by antibiotic-resistant bacteria, and destroying skin cancer cells. For example, AgNPs could be incorporated into wound

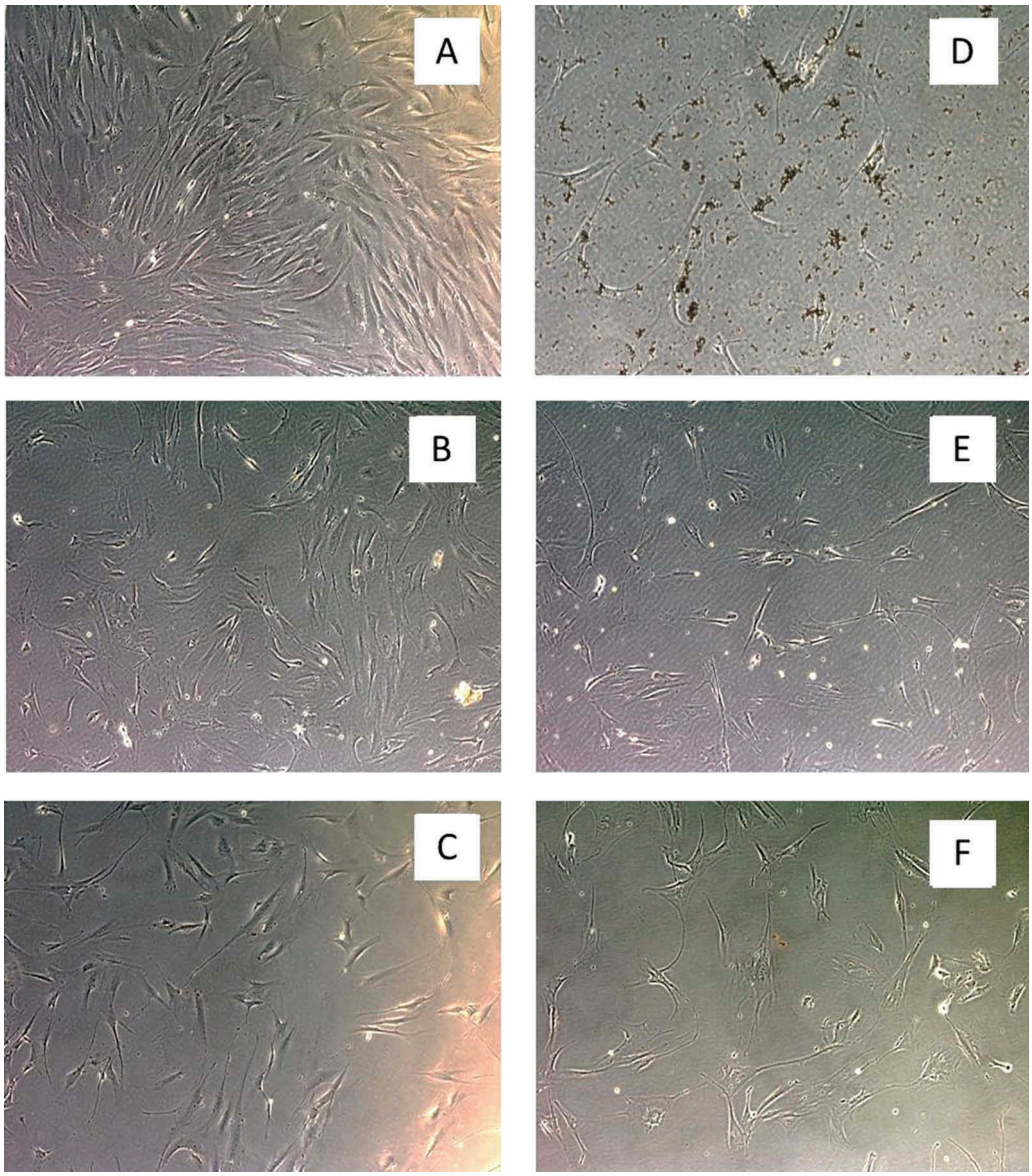

Figure 4. Images of optical microscopy of human skin fibroblasts (magnification ×200). (A) Intact cells, (B) cells subjected to hydrogen peroxide (H_2O_2), (C) cells exposed to spherical AgNPs and 2400 MHz EMF, (D) cells subjected to the treatment with graphene NPs and 7500 MHz EMF, (E) cells treated with triangular AgNPs and 900 MHz EMF and (F) cells exposed to colloidal AgNPs and 7500 MHz EMF.

dressings based on a macro-porous polymeric or silica matrix to fight wound pathogens. The controllable release of ROS from AgNPs will be triggered by external application of EMF. Such a system would allow the optimization of wound treatment, where AgNPs would prevent bacterial contamination of the affected area, while the polymeric matrix would provide a favorable environment for wound healing, including re-epithelization and control of wound exudate. However, further *in vivo* studies are required for the clinical implementation of the proposed system, which would combine bio-effects induced by AgNPs and EMF.

This study demonstrated the feasibility of utilizing of the combination of graphene-based nanomaterials and EMF for heat generation in tissues and organs. Such a technology provides an opportunity to develop novel thermosensitive graphene-based nanocarriers for effective targeted drug release or for tumor ablation. In the context of wound treatment, graphene could be incorporated into wound dressings to generate controllable therapeutic heat effect (upon exposure to EMF), thus improving local blood circulation and tissue repair. It must be also noted that apart from impressive thermo-conductive properties, in our experiments, GrNPs have also demonstrated exceptional ROS-protective properties. Such a feature might be

potentially extended to the protection of cells against ischemia and oxidative stress by means of graphene and EMF.

To summarize, our findings indicate the possibility to control cytotoxicity of AgNPs by using externally applied EMFs. To the best of our knowledge, the data on the combined effect of AgNPs, GrNPs and EMF have been reported here for the first time.

Acknowledgments

Authors would like to thank the company TMSpetsMash Ltd (Kyiv, Ukraine) for providing graphene samples. Special thanks to Dr. Vyacheslav Ogay from National Centre for Biotechnology (Astana, Kazakhstan) for providing cell culture, and to Dr. Timur Abdullin from Kazan Federal University (Russian Federation) for the discussion of the results. In addition, the authors also would like to acknowledge Professor Les Baillie and Professor Adrian Porch (Cardiff University, UK) for the idea of using electromagnetic radiation for the enhancement of the antibacterial properties of silver nanoparticles.

Disclosure Statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Funding

The study was supported by the grant of Ministry of Education and Science of the Republic of Kazakhstan "Nanoparticles-based wound dressings with microwave-enhanced antimicrobial function": [Grant Number 0113RK00784/N 698], the Royal Academy of Engineering grant "Wound dressings for malodorous chronic ulcers" (Newton UK-Kazakhstan Industry Academia Partnership Programme, UK, project IAPP/1516/12) and FP7 project IRSES-NanoBioMat (TA and SM) and FP7 project IRSES-ENSOR (GK).

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